Abnormal synthesis of cartilage-characteristic proteoglycan in azaserine-induced micromelial limbs

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Administration of azaserine (250 µg) to day-4 chick embryos *in ovo* was shown to induce micromelial limbs. In the present study, biosynthesis of cartilage-characteristic proteoglycan H (PG-H) as an index of limb chondrogenesis was examined in normal and micromelial hind limbs from day-7 chick embryos by biochemical and immunological methods. (1) Metabolic labelling of the micromelial limbs with [6-³H]-glucose and [³⁵S]sulphate, followed by analysis of labelled proteoglycans by glycerol-density-gradient centrifugation under dissociative conditions, showed a marked reduction in PG-H synthesis. (2) PG-H synthesized by micromelial limbs differed from that synthesized by normal limbs in possessing a slower sedimenting velocity and much lower amounts of chondroitin sulphates. (3) The amount of PG-H core protein in micromelial limbs was significantly decreased to about 19% on a per limb basis and about 42% on a per DNA basis of that in normal limbs, as determined by e.l.i.s.a. (4) The transition from PG-M to PG-H during limb formation was retarded in micromelial limbs as judged by an indirect immunofluorescence technique using antibodies against PG-M and PG-H. (5) The deficiency of incorporation of labelled glucose into chondroitin sulphate chains of PG-H in micromelial limbs was partially restored by using [6-³H]-glucosamine as a precursor, suggesting that the synthesis of UDP-*N*-acetylhexosamine, required for chondroitin sulphate chain synthesis of PG-H in micromelial limbs, was decreased. These results suggest that the reduction in the synthesis of PG-H as well as the production of an abnormal form of PG-H during a critical period of limb morphogenesis may be important factors in explaining the micromelia induced by azaserine.

INTRODUCTION

Some drugs occasionally induce skeletal malformations such as phocomelia and micromelia as a result of teratogenic side effects, and these effects have become serious problems [1]. The elucidation of the teratogenic mechanisms is of great concern.

Azaserine (*O*-diazooacetyl-L-serine), a L-glutamine antagonist, has been shown to be an experimental teratogen in chick embryos [2] and rats [3]. This drug, when given in a dose of 250 µg to day-4 chick embryos (stage 24, Hamburger & Hamilton [4]), causes micromelia [2,5]. It is known that glutamine analogues such as azaserine and 6-diazo-5-oxo-L-norleucine (DON) inhibit the biosynthesis of purines and glycosaminoglycans, presumably by blocking the glutamine-dependent steps in their biosynthetic pathways [6–10], although synthesis of protein and collagen appears to be unaffected [9]. However, the causal relationship between the inhibitory effects of azaserine and the sequence of molecular events leading to micromelia is not fully understood.

Recent studies by Suzuki and co-workers have shown that chick-embryo epiphyseal cartilage at early stages of development synthesizes at least four proteoglycan species: cartilage-characteristic proteoglycan (PG-H) [11,12], PG-L₁, PG-L₂ [13,14] and PG-M [15], which are

analogous in having glycosaminoglycan side chains of the chondroitin (dermatan) sulphate type but which are different from one another in the structures of the core proteins. PG-M, a large chondroitin sulphate proteoglycan, is synthesized at a maximum rate before chondrogenesis in chick limb buds (stages 22–23). However, there is a great decrease in the synthesis of PG-M with further development of cartilage [15].

In this report we have compared the structures of the cartilage-characteristic proteoglycan, PG-H, produced by the normal and the micromelial hind limbs, using an antiserum prepared against PG-H. We found that abnormalities of PG-H biosynthesis in the micromelial limbs were induced by azaserine.

MATERIALS AND METHODS

Materials

Freshly laid fertilized eggs of White Leghorn chickens were obtained from Saitama Prefectural Poultry Experiment Station, Saitama, Japan, and were incubated at 38.5 °C in an electric incubator. On day 4 (96 h incubation, i.e. Hamburger–Hamilton stage 24 [4]) 250 µg of azaserine in 50 µl of sterilized distilled water was injected into the extra-embryonic coelom between the

Abbreviations used: PG-H, proteoglycan H; PG-M, proteoglycan M; DON, 6-diazo-5-oxo-L-norleucine; PBS/Tween, phosphate-buffered saline (0.15 M-sodium chloride/20 mM-sodium phosphate, pH 7.0) containing 0.05% Tween-20.

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inner shell membrane and the vascular splanchnopleure. Normal control eggs were given 50 μ l of sterile distilled water only. The eggs were incubated until day 7. On day 7 (Hamburger–Hamilton [4] stages 30–31), the limbs were excised from the root of the hind limbs of the azaserine-treated or normal embryos, and were used for subsequent experiments.

The following commercial materials were used: D-[6- 3 H]glucose (31 mCi/mmol), D-[6- 3 H]glucosamine hydrochloride (40 Ci/mmol), [methyl- 3 H]thymidine (25 Ci/mmol), [3 H]amino acid mixture (1 mCi/mmol), ACS II aqueous counting scintillant and NCS tissue solubilizer from Amersham International; [35 S]sulphuric acid (46 mCi/ml of 0.05 M-HCl solution, carrier-free) from Japan Atomic Energy Institute, Tokyo, Japan; medium 199 from Nissui Seiyaku Co., Ltd., Tokyo, Japan; streptomycin sulphate from Meiji Seika, Tokyo, Japan; penicillin from Banyu Co. Ltd., Tokyo, Japan; azaserine from Sigma Chemical Co., St. Louis, MO, U.S.A.; guanidine hydrochloride from Nakarai Chemical Ltd., Kyoto, Japan; pepstatin A from Peptide Institute Inc., Osaka, Japan; sodium deoxycholate from Difco Laboratories, Detroit, MI, U.S.A.; bovine albumin (crystallized, purity 100%) from ICN Nutrition Biochemicals, Cleveland OH, U.S.A.; Pronase E from Kaken Chemicals Co., Tokyo, Japan; chondroitinase ABC (EC 4.2.2.4) from *Proteus vulgaris*; standard unsaturated disaccharide kit and whale cartilage chondroitin 4-sulphate from Seikagaku Kogyo Co., Tokyo, Japan; Sepharose CL-6B from Pharmacia; Toyo filter paper (no. 51 A) from Toyo Roshi, Tokyo, Japan. All other reagents were of the highest grade commercially available.

Histological and histochemical methods

The limbs obtained were fixed in 1% cetylpyridinium chloride in 10% formalin at 4°C for between 24 and 96 h [16]. After fixation, the tissue specimens were dehydrated in graded ethanol series, cleared in xylene and embedded in paraffin wax. Sections of thickness 4 μ m were cut on a sliding microtome, affixed to glass slides, deparaffinized in xylene, hydrated through an ethanol series of descending concentrations and stained with either haematoxylin-eosin or Alcian blue (AB), pH 1.0 [17].

Labelling experiments

Dissected hind limbs from day-7 normal (about 300 mg wet weight/30 limbs) and azaserine-treated (about 57 mg wet weight/30 limbs) embryos were preincubated for 20 min in a 10 ml conical flask at 37°C in an O₂/CO₂ (19:1) atmosphere with 2 ml of Krebs' improved Ringer 1 medium (pH 7.4) [18] without glucose containing antibiotics (penicillin, 100 units/ml and streptomycin, 100 μ g/ml) and then incubated for another 2 h in the presence of 75 μ Ci of H₂³⁵SO₄ and 75 μ Ci of D-[6- 3 H]glucose. After the end of the incubation, the limbs were rinsed with three 5 ml changes of ice-cold Krebs' improved Ringer 1 medium and the resulting labelled limbs were used for subsequent experiments. Incorporation of 3 H or 35 S radioactivity into the limbs was linear for up to 6 h of incubation. In other incubation experiments using D-[6- 3 H]glucosamine and H₂³⁵SO₄ as precursors, the labelling conditions were the same as described above except for the addition of 70 μ Ci of

D-[6- 3 H]glucosamine and 70 μ Ci of H₂³⁵SO₄ to the medium.

In the incubation with the [3 H]amino acid mixture, the same labelling conditions were used except for the addition of 2 μ Ci of [3 H]amino acid mixture to Krebs' improved Ringer 1 medium (pH 7.4) [18]. The resulting labelled limbs were placed in 1 ml of ice-cold distilled water and ground in a chilled mortar. The homogenate, after 1 ml of ice-cold 20% trichloroacetic acid was added to a final concentration of 10%, was centrifuged at 8000 g for 20 min at 4°C. The precipitate was washed three times with 10% trichloroacetic acid and then collected by centrifugation. The pellet was dissolved in 1 ml of 0.3 M-NaOH and then neutralized with 1 ml of 0.3 M-HCl. An aliquot of the sample was assayed for radioactivity.

For incubation with [3 H]thymidine as a precursor for DNA synthesis, the dissected normal and micromelial hind limbs (5 limbs) from day-5–7 chick embryos were preincubated for 20 min in a 10 ml conical flask at 37°C in an O₂/CO₂ (19:1) atmosphere with 2 ml of Medium 199 containing antibiotics (penicillin, 100 units/ml and streptomycin, 100 μ g/ml) and then incubated for another 2 h in the presence of 25 μ Ci of [methyl- 3 H]thymidine. After the end of incubation, the labelled limbs were rinsed with three 5 ml changes of ice-cold Medium 199, placed in 1 ml of ice-cold distilled water and ground in a chilled mortar. The homogenate was treated with 1 ml of 20% trichloroacetic acid at a final concentration of 10%, and the resulting precipitate was collected by centrifugation at 2000 g for 10 min at 4°C. The pellet was washed three times with 10% trichloroacetic acid. To the pellet was added 1 ml of ethanol/diethyl ether (3:1, v/v), and the mixture was kept for 30 min at room temperature. The precipitate was then collected by centrifugation at 8000 g for 10 min at 25°C. The supernatant was discarded and the ethanol/ether extraction procedure was repeated twice. The resulting pellet was dissolved with 1 ml of NCS tissue solubilizer, and an aliquot was assayed for radioactivity.

Extraction of radioactive proteoglycans

Labelled proteoglycans were extracted from normal or micromelial hind limbs by the method described previously [19,20]. Briefly, the labelled tissues were extracted with 5 vol. (about 1 ml) of cold extraction solution consisting of 4 M-guanidine hydrochloride/0.05 M-Tris buffer (pH 8.0) containing the following protease inhibitors: 10 mM-N-ethylmaleimide, 10 mM-disodium EDTA, 1 mM-phenylmethanesulphonyl fluoride and 0.36 mM-pepstatin (solution A). The extracts were used as crude proteoglycan preparations for further separation of the cartilage-characteristic PG-H by zonal sedimentation on a glycerol gradient in 4 M-guanidine hydrochloride. About 80% of the total labelled proteoglycans in normal or micromelial limbs were extracted by this procedure.

Glycerol-density-gradient centrifugation of proteoglycans

Rate zonal sedimentation of proteoglycans was performed as described previously [20]. Briefly, an 0.8 ml portion of the crude extracted proteoglycan preparations with solution A was layered on a 15 ml gradient of glycerol (10–35% in solution A) in a centrifugal polyallomer tube, at the bottom of which 0.5 ml of 50% glycerol in solution A had been placed. The tube was

centrifuged in an SRP-28SA1 rotor in a Hitachi 65 P ultracentrifuge at 70000 *g* at 20 °C for 28 h. Fractions (0.5 ml) were collected and used for the measurement of radioactivity. The recovery of total labelled proteoglycans from both normal and micromelial samples after density-gradient centrifugation was more than 90 %.

Preparation of anti-PG-H and PG-M antibodies

Anti-PG-H antibody and anti-PG-M antibody were prepared as described previously [15,20].

Immunofluorescence study of limbs

For immunofluorescent staining and microscopy, frozen sections of normal or micromelial hind limbs at stages 30–31 (day-7 chick embryos) were prepared as described previously [20]. The sections were treated with anti-PG-M or anti-PG-H antibody and then with fluorescein isothiocyanate-conjugated goat antibodies to rabbit immunoglobulins (Miles-Yeda, Rohovot, Israel), as previously described [15,20]. In control experiments, parallel sections were treated with normal rabbit immunoglobulin prepared from a non-immunized rabbit. The treated sections were viewed with an Olympus BH₂RFL epi-illuminated fluorescence microscope (Olympus Kogaku Co., Tokyo, Japan).

Immunoprecipitation of cartilage-characteristic PG-H with anti-PG-H serum

Labelled PG-H recovered from the glycerol-density-gradient fractions in 4 M-guanidine hydrochloride was precipitated with 3 vol. of ice-cold 95 % (v/v) ethanol containing 1.3 % (w/v) potassium acetate. The precipitate was dissolved in 1 ml of 50 mM-Tris/HCl buffer (pH 7.4) containing 0.15 M-NaCl, 0.25 % Triton X-100, 0.15 % bovine serum albumin and protease inhibitors (10 mM-*N*-ethylmaleimide, 10 mM-disodium EDTA, 1 mM-phenylmethanesulphonyl fluoride and 0.36 mM-pepstatin) (solution D) with gentle shaking at 4 °C overnight. The solution was immunoprecipitated by the anti-PG-H antiserum as a first antibody, followed by the second antibody, goat anti-(rabbit IgG) antiserum (17.5 mg of antibody/ml; Miles Laboratories Inc., Naperville, IL, U.S.A.), and then the immunoprecipitate was washed as described previously [20]. The resulting washed immunoprecipitate was dissolved in 1 ml of solution A and re-centrifuged on a glycerol-density gradient as above. Between 81 and 88 % of the total radioactivity of the PG-H fractions from normal or micromelial limbs was recovered in the immunoprecipitate.

E.l.i.s.a.

E.l.i.s.a. was done as described previously [21]. Briefly, antigen (PG-H standard was prepared as described previously [11]) was diluted into Vollers buffer [20 mM-sodium carbonate, pH 9.6, containing 0.02 % (w/w) sodium azide] to the desired concentration (10 µg of protein/ml). An aliquot (200 µl) was pipetted into each microtitre well (polyvinyl 96 U-type microtitre plate, Costar 2797) and allowed to adsorb at 4 °C overnight. The plates were then rinsed three times with 200 µl per well of phosphate-buffered-saline/Tween-20 (PBS/Tween) [0.15 M-sodium chloride/20 mM-sodium phosphate/0.05 % Tween-20, pH 7.0] (step 1). Anti-PG-H antiserum was diluted into PBS/Tween (1:3000 dilution) and aliquots were added to the microtitre plates.

After 30 min of gentle shaking at room temperature, the plates were washed three times with 200 µl of PBS/Tween per well (step 2). Horseradish peroxidase-conjugated goat anti-(rabbit IgG) as the second antibody was diluted with PBS/Tween (1:1000 dilution) and aliquots were added to the microtitre plates. After 60 min of gentle shaking at room temperature, the plates were washed three times with 200 µl of PBS/Tween per well (step 3). The enzyme substrate [0.1 mg of *o*-phenylenediamine/ml/0.03 % (v/v) H₂O₂] was added and the colour was allowed to generate for 60 min, after which the reaction was stopped by adding 50 µl of 8 M-H₂SO₄ (step 4). The brown colour produced was measured spectrophotometrically at 492 nm using a microtitre plate reader (plate analyser ETY-III; Oriental Instruments Ltd., Tokyo, Japan).

For the assay of PG-H core protein, the hind limbs (30 limbs) of day-7 normal and azaserine-treated embryos were extracted with 1 ml of solution A and the extracts were centrifuged on a glycerol-density-gradient under dissociative conditions as above. The faster sedimenting proteoglycan fractions (5–22) were precipitated with 3 vol. of ice-cold 95 % (v/v) ethanol containing 1.3 % (w/v) potassium acetate. After centrifugation, the precipitates were sonicated at 50 W for 10 s in 1 ml of Vollers buffer. The suspensions were used for e.l.i.s.a.

For the inhibition test, the measured dilution of PG-H antiserum was added to a test solution containing unknown amounts of PG-H core protein and incubated overnight at 4 °C for the binding. This mixture was then added to the antigen-coated plate in step 2 above. PG-H in the test solution blocks binding of part of the antibody to the antigen-coated plate and thereby inhibits the generation of colour in step 4.

Preparation of radioactive carbohydrate chains from PG-H

Carbohydrate chains from PG-H were prepared after the treatment of PG-H with 0.05 M-NaOH/0.1 M-NaBH₄ and, after neutralization, with Pronase E as described previously [20].

Gel chromatography

Molecular size distributions of the radioactive carbohydrate chains from PG-H of normal or micromelial limbs were determined by gel chromatography on a column (1.8 cm × 65–70 cm) of Sepharose CL-6B. The column was equilibrated and eluted with 0.2 M-pyridine/0.2 M-acetic acid (pH 5.0) at a flow rate of 6 ml/h at 4 °C. Fractions (3 ml) were collected and assayed directly for radioactivity. Whale-cartilage chondroitin 4-sulphate (*M_r* 38000) was used as a standard to determine molecular sizes.

Other methods

Uronic acid was determined by the carbazole method of Bitter & Muir [22], with glucuronolactone as a standard.

DNA was extracted by the methods of Schmidt & Thannhauser [23] and Schneider [24], and the amount was determined by the method of Burton [25].

Protein content was estimated by Lowry's method [26] with bovine serum albumin as a standard.

Chondroitinase ABC digestion and paper chromatography were done as described by Saito *et al.* [27].

RESULTS

Morphological and biochemical alterations in micromelial hind limbs induced by azaserine

When a dose of 250 μg of azaserine was administered to day-4 chick embryos *in ovo*, malformations such as a reduction in body size, shortening of the limbs (micromelia) and beak defects were observed in the embryos by day 7. However, administration of azaserine (250 $\mu\text{g}/\text{egg}$) to day-7 chick embryos *in ovo* did not induce micromelial limbs. These observations are consistent with results in the literature [2,5].

Histological observations showed in the limb cartilage of control chick embryos on day 7, chondrocytes were sphere- or ellipsoid-shaped and surrounded by intracellular matrix which was relatively abundant and which exhibited vivid basophilia (Fig. 1a) and alcianophilia (Fig. 1b). Likewise, lacunar borders were well differentiated from the rest of the intercellular matrix (Fig. 1b). In contrast, in the limb cartilage of azaserine-treated chick embryos 3 days after treatment, chondrocytes were small, thin and spindle-shaped (Fig. 1c). The chondrocytes were more numerous in a definite volume of the matrix (Fig. 1c), as compared with those in the control tissues (Fig. 1a). In addition, the intercellular matrix showed feeble basophilia (Fig. 1c) and relatively weak alcianophilia (Fig. 1d). Furthermore, lacunar borders could not be identified at all in the experimental cartilage tissue (Fig. 1d).

The wet weights of whole body and of hind limbs, and the amounts of both DNA and uronic acid of hind limbs, decreased significantly in day-4 azaserine-injected embryos from days 5–7 compared with those in the untreated embryos (Table 1). The micromelia was observed in the hind limbs, as judged by the wet weight ratio of hind limbs to body.

Alterations in DNA and protein synthesis of the micromelial hind limbs induced by azaserine

Tables 2 and 3 show the alterations in DNA and protein synthesis respectively in hind limbs from normal and azaserine-treated embryos.

Both the total radioactivity (per 10 limbs) and the specific radioactivity (per μg of DNA) of [^3H]thymidine in the trichloroacetic acid-insoluble DNA fraction were markedly decreased in the micromelial limbs, as compared with normal limbs, from day 5 to day 7, i.e. 1–3 days after azaserine injection. In contrast, the incorporation of the [^3H]amino acid mixture into the trichloroacetic acid-insoluble fraction of micromelial limbs on day 7 was about 55% (47.5×10^4 d.p.m./2 h per 10 limbs) and about 121% (2.3×10^3 d.p.m./2 h per μg DNA) respectively of the normal. These results indicate that DNA synthesis was inhibited in the azaserine-induced micromelial limbs as compared with the normal limbs, whereas the protein synthetic activity in micromelial limbs was not significantly affected on a per DNA basis, but decreased on a whole-limb basis.

Distribution patterns of PG-M and PG-H in normal and micromelial hind limbs

Distribution of PG-M and PG-H between day-7 normal and micromelial hind limbs was compared by immunofluorescent staining with anti-PG-M and anti-PG-H antibodies. Limb cartilages from both normal and azaserine-treated embryos were stained with the antibodies directed against the core protein of PG-H (Figs. 2a,b,d). However, in azaserine-treated limbs, the fluorescence of PG-H showed an uneven distribution and indicated abnormal shape of the cartilage (Fig. 2d). In contrast, the fluorescence of PG-M was observed in limbs from azaserine-treated embryos (Fig. 2e), but was

Fig. 1. Morphological alterations in micromelial hind limbs induced by azaserine

The sections of normal (a, b) and micromelial (c, d) hind limbs at day 7: (a) and (c) were stained with haematoxylin-eosin; (b) and (d) were stained with Alcian Blue, pH 1.0. For further experimental details, see the text. Magnification $\times 88$.

Table 1. Developmental alterations in wet weight and amounts of DNA and uronic acid of the hind limbs, and body weight, in normal and azaserine-treated embryos on days 5–7

The values represent the means $\pm$ S.D. for numbers of experiments given in parentheses. For further experimental details see the text. Significantly different from 'normal' value: * $P < 0.05$; † $P < 0.01$; ‡ $P < 0.001$.

	Treatment	Day 5	Day 6	Day 7
Wet weight (mg/10 limbs)	Normal	21.3 $\pm$ 5.2 (4)	79.2 $\pm$ 4.2 (4)	175.2 $\pm$ 14.4 (5)
	Azaserine	3.8 $\pm$ 0.9 (3)†	11.6 $\pm$ 3.4 (4)‡	19.3 $\pm$ 1.4 (4)‡
Body weight (mg/embryo)	Normal	124.9 $\pm$ 15.2 (8)	337.3 $\pm$ 60.6 (18)	536.5 $\pm$ 77.2 (12)
	Azaserine	77.6 $\pm$ 14.7 (8)‡	117.4 $\pm$ 23.8 (8)‡	177.3 $\pm$ 15.4 (8)‡
Limb weight/body weight	Normal	0.17	0.23	0.33
	Azaserine	0.05	0.10	0.11
DNA (μ g/10 limbs)	Normal	235.6 $\pm$ 8.4 (4)	341.7 $\pm$ 5.9 (3)	553.3 $\pm$ 95.6 (4)
	Azaserine	179.2 $\pm$ 15.6 (3)†	229.2 $\pm$ 23.6 (3)†	288.5 $\pm$ 55.1 (4)†
Uronic acid (μ g/10 limbs)	Normal	5.8 $\pm$ 1.4 (3)	17.8 $\pm$ 6.8 (3)	30.7 $\pm$ 6.4 (3)
	Azaserine	2.4 $\pm$ 0.2 (3)*	4.3 $\pm$ 0.6 (3)*	7.6 $\pm$ 2.0 (3)†

Table 2. Developmental alterations in DNA synthesis in hind limbs from normal and azaserine-treated embryos

Hind limbs from day-5, day-6 and day-7 embryos were incubated in 2 ml of Medium 199 containing 25 μ Ci of [*methyl*- 3 H]thymidine for 2 h at 37 °C. For further details, see the text. The total radioactivity (T) and the specific radioactivity (S) are expressed as $10^{-5} \times$ (d.p.m./10 limbs) and $10^{-3} \times$ (d.p.m./ μ g of DNA) respectively. The values represent the means $\pm$ S.D. ($n = 3$). Significantly different from normal value: * $P < 0.05$; † $P < 0.01$.

Treatment		Day 5	Day 6	Day 7
Normal	T	10.0 $\pm$ 0.8	17.3 $\pm$ 1.2	26.5 $\pm$ 3.6
	S	4.2 $\pm$ 0.3	5.0 $\pm$ 0.4	4.8 $\pm$ 0.6
Azaserine	T	5.9 $\pm$ 0.5†	7.5 $\pm$ 2.4†	7.4 $\pm$ 2.5†
	S	3.3 $\pm$ 0.2*	3.3 $\pm$ 0.9*	2.5 $\pm$ 0.8*

Table 3. Protein synthesis in hind limbs from day-7 normal and azaserine-treated embryos

Normal and micromelial hind limbs from day-7 embryos were incubated in 2 ml of Krebs' improved Ringer 1 medium (pH 7.4) [18] containing 2 μ Ci of a [3 H]amino acid mixture for 2 h at 37 °C. The trichloroacetic acid-insoluble materials were used for analyses. For further details, see the text. The total radioactivity (T) and the specific radioactivity (S) are expressed as $10^{-4} \times$ (d.p.m./10 limbs) and $10^{-3} \times$ (d.p.m./ μ g of DNA) respectively. The values represent the means $\pm$ S.D. ($n = 3$). Significantly different from normal value: * $P < 0.05$.

Treatment	Protein synthesis	
	T	S
Normal	87.1 $\pm$ 10.5	1.9 $\pm$ 0.2
Azaserine	47.5 $\pm$ 8.2*	2.3 $\pm$ 0.4

virtually absent in normal limbs (Fig. 2c). These results suggest that the transition from PG-M to PG-H during cartilage development is retarded by the azaserine treatment.

Fig. 2. Indirect immunofluorescent staining of normal (a, b, c) and azaserine-induced micromelial (d, e) hind limbs of day-7 chick embryos

Sections (a), (b) and (d) were stained with anti-(cartilage-characteristic PG-H) antibody (magnifications: a and d $\times 63$; b $\times 167$). Sections (c) and (e) were stained with anti-PG-M antibody ($\times 167$).

PG-H synthesis in normal and micromelial hind limbs

The crude [3 H]glucose- and [35 S]sulphate-labelled proteoglycans obtained from normal and azaserine-induced micromelial hind limbs of day-7 embryos (15 and 30 limbs respectively) were centrifuged on the glycerol-density-gradient under dissociative conditions as shown in Fig. 3. The faster sedimenting proteoglycan fractions, which peaked at fractions 13–14, were the major component of the extract from normal limbs. This peak corresponds to a typical PG-H population previously

Fig. 3. Zone sedimentation profiles of [^3H]glucose- and [^{35}S]sulphate-labelled proteoglycans synthesized by normal (a) and micromelial (b) limbs

[^3H]Glucose- and [^{35}S]sulphate-labelled proteoglycans, obtained from 15 hind limbs of day-7 normal (a) or from 30 hind limbs of day-7 azaserine-treated (b) chick embryos, were layered on a 15 ml glycerol gradient (10–35%) in solution A and centrifuged at 70000 g for 28 h at 20 °C. Fractions (0.5 ml) were collected and assayed for radioactivity. Authentic cartilage-characteristic PG-H (about 2 mg wet weight/gradient) from epiphyseal cartilage of day-12 chick embryos was centrifuged under the same conditions as a reference, and uronic acid per fraction was assayed [22] (c). For further experimental details, see the text.

described [20]. In the extracts from micromelial limbs, however, a slower sedimenting population, with a peak at fractions 17–19, was the predominant component. Both of the major fractions (indicated by the bars above the curves in Fig. 3) from normal and micromelial limbs could be immunoprecipitated by the antibodies against PG-H, suggesting that they contained the antigenic determinant, i.e. the core protein of PG-H. The immunoprecipitates were again centrifuged on a glycerol-density gradient under dissociative conditions. As shown in Fig. 4, the peak from the micromelial immunoprecipitate gave a peak which was again shifted to slower sedimenting fractions, compared with that of the normal. The difference in sedimentation velocity may be due

Fig. 4. Zone sedimentation profiles of [^3H]glucose- and [^{35}S]sulphate-labelled PGs immunoprecipitated with anti-PG-H serum from extracts of normal (a) and micromelial (b) limbs

Experimental conditions for the glycerol-gradient centrifugation and immunoprecipitation are described in the legend to Fig. 3 and in the text. Extracts from 15 (a) and 30 (b) limbs were used.

to a decrease in the relative proportion of chondroitin sulphate chains to core protein in the micromelial PG-H (see below).

The activity of PG-H synthesis was compared in normal and micromelial hind limbs from day-7 embryos. Limbs were metabolically labelled with [^3H]glucose and [^{35}S]sulphate. In the micromelial limbs, the incorporation of [^3H]glucose and [^{35}S]sulphate into PG-H fraction (Fig. 4) occurred at about 50% (8.0×10^4 d.p.m./2 h per 10 limbs) and 35% (3.9×10^4 d.p.m./2 h per 10 limbs) respectively of the normal rates. These results suggest that the rate of biosynthesis of PG-H is much lower in micromelial limbs than in normal limbs.

[^3H]Glucose- and [^{35}S]sulphate-labelled carbohydrate chains were prepared after NaBH_4 /alkali treatment and Pronase E digestion of the immunoprecipitates with anti-PG-H antibody, and then were subjected to gel chromatography on Sepharose CL-6B (Fig. 5). Normal samples yielded two distinct peaks, the high- M_r fraction (fraction H, K_{av} 0.30) and the low- M_r fraction (fraction L, K_{av} 0.76) (see [20]). The elution position of fraction H corresponded to that of chondroitin 4-sulphate from whale cartilage (M_r 38000; K_{av} 0.30). Fraction H was identified as chondroitin sulphates as judged by the complete digestion with chondroitinase ABC. On the

Fig. 5. Sepharose CL-6B chromatography of [^3H]glucose- and [^{35}S]sulphate-labelled carbohydrate chains released from PG-H synthesized by normal (a) and micromelial (b) limbs

The pooled PG-H materials (indicated by the bars above the curve in Fig. 4) were treated with 0.05 M-NaOH/0.1 M-NaBH₄ and then digested with Pronase E. The resulting radioactive carbohydrate chains were subjected to a column (1.8 cm $\times$ 70 cm) of Sepharose CL-6B and eluted with 0.2 M-pyridine/0.2 M-acetic acid (pH 5.0). Fractions (3 ml) were collected and assayed for radioactivity.

other hand, azaserine-induced micromelial samples yielded only fraction L with a significant decrease in radioactivity.

Both fractions L were separated into three peaks (low- M_r chondroitin sulphates plus keratan sulphates, *N*-linked oligosaccharides and *O*-linked oligosaccharides) by gel chromatography on Sephadex G-50 as described previously [20] (results not shown). However, they were not characterized further in this study.

These results suggest that proteoglycan in azaserine-induced micromelial limbs corresponded to PG-H in terms of the immunological properties, but was abnormal in having few high- M_r chondroitin sulphate chains.

Decrease in the amount of PG-H core protein in the micromelial hind limbs measured by e.l.i.s.a.

To examine whether or not the decrease of PG-H synthesis in the micromelial limbs induced by azaserine was due to reduced synthesis of the core protein, the amounts of PG-H core protein in normal and micromelial hind limbs from day-7 embryos were estimated by e.l.i.s.a. As shown in Table 4, the content of PG-H core protein in micromelial limbs was about 19% (87.2 ± 24.0 $\mu\text{g}/30$ limbs) or about 42% (142.4 ± 39.1 $\mu\text{g}/\text{mg}$ of DNA) compared with the normal. These results indicate that the synthesis of PG-H core protein in micromelial limbs was significantly decreased both on a per limb basis and on a per DNA basis.

Table 4. Amounts of PG-H core protein in hind limbs of day-7 normal and azaserine-treated embryos

The amounts of PG-H core-protein from normal and micromelial hind limbs of day-7 embryos were estimated by e.l.i.s.a.. For further experimental details, see the text. The values represent the means $\pm$ S.D. ($n = 3$). Significantly different from normal value: * $P < 0.05$; † $P < 0.01$.

Treatment	PG-H core protein	
	($\mu\text{g}/30$ limbs)	($\mu\text{g}/\text{mg}$ of DNA)
Normal	471.8 ± 99.7	340.0 ± 71.9
Azaserine	87.2 ± 24.0 †	142.4 ± 39.1 *

DISCUSSION

The results presented in this paper indicate that PG-H synthesis in azaserine-induced micromelial limbs is retarded compared with that in normal limbs (Figs. 2 and 4; Table 4), and that the PG-H synthesized in micromelial limbs differs from that in normal limbs in possessing a slower sedimenting velocity (Figs. 3 and 4) and few chondroitin sulphate chains (Fig. 5). The content of PG-H core protein in micromelial limbs was markedly decreased to about 19% on a per limb basis and to about 42% on a per DNA basis, compared with that of normal limbs (Table 4). On the other hand, total protein synthesis activity on a per DNA basis in the micromelial limbs (day 7) was not affected (Table 3). Therefore, the inhibition of PG-H core-protein synthesis in azaserine-induced micromelial limbs may be a specific effect rather than a reflection of general inhibition of protein synthesis.

The glutamine analogues azaserine and DON are known to block the utilization of L-glutamine in transamination reactions, and thus they are considered to interfere with the biosyntheses of both glycosaminoglycans [6–10] and purines [8]. Glycosaminoglycan synthesis is probably blocked by the inhibition of the specific aminotransferase which catalyses the transfer of amide nitrogen from L-glutamine to fructose 6-phosphate for the synthesis of glucosamine 6-phosphate [8–10]. Thus we examined whether or not the deficiency of incorporation of labelled glucose into chondroitin sulphate chains (fraction H) of PG-H in micromelial limbs from day-7 embryos (Fig. 5) was really due to the inhibition of aminotransferase by azaserine, by labelling limbs with [$^6\text{-}^3\text{H}$]glucosamine and [^{35}S]sulphate. A gel chromatogram (Fig. 6) of the labelled carbohydrates obtained from PG-H in day-7 micromelial limbs on Sepharose CL-6B showed that chondroitin sulphate chains of PG-H (corresponding to fraction H) could be produced even in the micromelial limbs if [$^6\text{-}^3\text{H}$]glucosamine instead of [$^6\text{-}^3\text{H}$]glucose was used as a precursor. These results suggest that azaserine blocks the aminotransferase and thereby decreases the synthesis of UDP-*N*-acetylhexosamine in the micromelial limbs. These findings from our teratogenic system are consistent with those obtained from L-glutamine-deficient chicken articular cartilage [28] and a DON-treated cultured chondrocyte [10] system *in vitro*. Thus one possible mechanism for azaserine action in teratogenic systems is the creation of a glutamine deficiency which reduces the concentration of glucosamine 6-phosphate available for chondroitin sul-

Fig. 6. Sephadex CL-6B chromatography of [^3H]glucosamine- and [^{35}S]sulphate-labelled carbohydrate chains released from PG-H synthesized by normal (a) and micromelial (b) limbs

The pooled PG-H materials immunoprecipitated with anti-PG-H antiserum after the second glycerol density-gradient centrifugation were treated with 0.05 M-NaOH/0.1 M-NaBH₄ and then digested with Pronase E. The resulting radioactive carbohydrate chains were subjected to a column (1.8 cm $\times$ 70 cm) of Sephadex CL-6B and eluted with 0.2 M-pyridine/0.2 M-acetic acid (pH 5.0). Fractions (3 ml) were collected and assayed for radioactivity. PG-H was obtained from 19 (a) or 26 (b) limbs. For further experimental details, see the text.

phate synthesis. However, this mechanism would not explain why the marked deficiency in synthesis of chondroitin sulphate chains (fraction H) rather than fraction L of PG-H was observed in the micromelial limbs. Although the termination mechanisms of glycosaminoglycan chains have not yet been elucidated, it has been suggested that chondroitin-sulphate chain length is dependent on the ratio between available acceptor (protein core) and the metabolic precursors or the chain-elongating transferase enzymes [29,30]. Therefore, the occurrence of short chondroitin sulphate chains (fraction L) in micromelial PG-H may be due to the relative lack of endogenous hexosamine, specifically UDP-*N*-acetylhexosamine, caused by azaserine treatment [10].

Indeed, the lower values for DNA content and DNA synthesis in azaserine-induced micromelial limbs (Tables 1 and 2) suggest that azaserine-induced micromelia may reflect an azaserine block of glutamine-dependent steps in purine synthesis. However, in this study, it is difficult to estimate how the inhibition of DNA synthesis by azaserine leads to the specific interference with PG-H core protein gene expression during limb formation [31].

Kimata *et al.* [15] have shown that PG-M is synthesized before chondrogenesis in the limb buds of chick embryos

(stage 22–23), and it plays an important role in the cell condensation process of chondrogenesis. The present finding that the immunofluorescence of PG-M still persisted in azaserine-treated limbs (day 7) (Fig. 2) suggests that there was the retardation in the expression of PG-H as a biochemical index of limb chondrogenesis in micromelial limbs.

The present study provides evidence for a reduction in the biosynthesis of PG-H resulting from the inhibition of PG-H core protein production, as well as a limitation in UDP-*N*-acetylhexosamine synthesis. Also, an abnormal form of PG-H is produced in azaserine-induced micromelial limbs during limb formation in chick embryos. Considering our previous results obtained with micromelial limbs induced by 6-aminonicotinamide [19,20], the retardation of chondrogenesis and the abnormalities of PG-H synthesis at a critical stage of limb morphogenesis must be key factors resulting in both growth retardation and disordered formation of cartilage.

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REFERENCES

1. Schardein, J. L. (1976) *Drugs as Teratogens*, CRC Press, Inc., Cleveland, Ohio
2. Dagg, C. P. & Karnofsky, D. A. (1955) *J. Exp. Zool.* **130**, 555–572
3. Murphy, M. L. & Karnofsky, D. A. (1956) *Cancer* **9**, 955–962
4. Hamburger, V. & Hamilton, H. (1951) *J. Morphol.* **88**, 49–92
5. Murphy, M. L., Dagg, C. P. & Karnofsky, D. A. (1957) *Pediatrics* **19**, 701–714
6. Rando, R. R. (1975) *Biochem. Pharmacol.* **24**, 1153–1160
7. Telser, A., Robinson, H. C. & Dorfman, A. (1965) *Proc. Natl. Acad. Sci. U.S.A.* **54**, 912–919
8. Greene, R. M. & Kochhar, D. M. (1975) *J. Embryol. Exp. Morphol.* **33**, 355–370
9. Linsenmayer, T. F. & Kochhar, D. M. (1979) *Dev. Biol.* **69**, 517–528
10. Clark, C. C., Richards, C. F., Pacifici, M. & Iozzo, R. V. (1987) *J. Biol. Chem.* **262**, 10229–10238
11. Oike, Y., Kimata, K., Shinomura, T., Nakazawa, K. & Suzuki, S. (1980) *Biochem. J.* **191**, 193–207
12. Oike, Y., Kimata, K., Shinomura, T., Suzuki, S., Takahashi, N. & Tanabe, K. (1982) *J. Biol. Chem.* **257**, 9751–9758
13. Shinomura, T., Kimata, K., Oike, Y., Noro, A., Hirose, N., Tanabe, K. & Suzuki, S. (1983) *J. Biol. Chem.* **258**, 9314–9322
14. Shinomura, T., Kimata, K., Oike, Y., Maeda, N., Yano, S. & Suzuki, S. (1984) *Dev. Biol.* **103**, 211–220
15. Kimata, K., Oike, Y., Tani, K., Shinomura, T., Yamagata, M., Uritani, M. & Suzuki, S. (1986) *J. Biol. Chem.* **261**, 13517–13525
16. Spicer, S. S., Horn, R. G. & Leppi, T. J. (1967) in *The Connective Tissue* (Wagner, B. M. & Smith, D. E., eds.), pp. 251–303, Williams and Wilkins, Baltimore

17. Lev, R. & Spicer, S. S. (1964) *J. Histochem. Cytochem.* **12**, 309
18. Krebs, H. A. (1950) *Biochim. Biophys. Acta* **4**, 249–269
19. Honda, A., Iwama, M., Umeda, T. & Mori, Y. (1982) *J. Biochem. (Tokyo)* **91**, 1959–1970
20. Honda, A., Kazuno, S., Mori, Y., Kimata, K. & Suzuki, S. (1987) *Biochem. J.* **246**, 745–753
21. Rennard, S. I., Kimata, K., Dusemund, B., Barrach, H. J., Wilczek, J., Kimura, J. H. & Hascall, V. C. (1981) *Arch. Biochem. Biophys.* **207**, 399–406
22. Bitter, T. & Muir, H. (1962) *Anal. Biochem.* **4**, 330–334
23. Schmidt, G. & Thannhauser, S. J. (1945) *J. Biol. Chem.* **161**, 83–89
24. Schneider, W. C. (1946) *J. Biol. Chem.* **164**, 747–751
25. Burton, K. (1956) *Biochem. J.* **62**, 315–323
26. Lowry, O. H., Rosebrough, N. J., Farr, A. L. & Randall, R. J. (1951) *J. Biol. Chem.* **193**, 265–275
27. Saito, H., Yamagata, T. & Suzuki, S. (1968) *J. Biol. Chem.* **243**, 1536–1542
28. Speight, G., Handley, C. J. & Lowther, D. A. (1978) *Biochim. Biophys. Acta* **540**, 238–245
29. Lohmander, S., Madsen, K. & Hinek, A. (1979) *Arch. Biochem. Biophys.* **192**, 148–157
30. Kato, Y., Kimata, K., Ito, K., Karasawa, K. & Suzuki, S. (1978) *J. Biol. Chem.* **253**, 2784–2789
31. Stirpe, N. S., Argraves, W. S. & Goetinck, P. F. (1987) *Dev. Biol.* **124**, 77–81

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